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(54) Title: METHOD OF DIAGNOSING A MYCOBACTERIAL DISEASE AND IMMUNOASSAY KIT		
(57) Abstract A method of diagnosing of mycobacterial disease in a patient typically caused by <i>Mycobacterium tuberculosis</i> (TBC) or <i>Mycobacterium avium</i> complex (MAC), is disclosed. In a sample of feces, sputum or urine from said patient, the presence of a mycobacterial antigen selected from the group consisting of lipoarabinomannans (LAM), arabinomannans (AM), and fragments of LAM and AM, is determined, e.g. by the use of polyclonal or monoclonal antibodies directed against said mycobacterial antigen, and an assay detecting antigen/antibody complexes formed. The sample of feces, sputum or urine can be pretreated by heat sterilization. Further, an immunoassay kit for use in the method of diagnosing, and a method of monitoring the effects of therapeutic treatment of a mycobacterial disease, are described.		

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Method of diagnosing a mycobacterial disease and immunoassay kit

5 The present invention relates to a method of diagnosing a mycobacterial disease, a method of monitoring the effects of therapeutic treatment of a mycobacterial disease and an immunoassay kit for diagnosing a mycobacterial disease in a patient.

10 **Background**

There are several types of mycobacteria causing diseases in man, and the most important ones, which are pathogenic to humans, belong to the group *Mycobacterium tuberculosis* (TBC) and *Mycobacterium avium* complex (MAC).

15 These bacteria have different, but closely related carbohydrate antigens on their cell walls, namely lipoarabinomannans (LAM), and arabinomannans (AM). One method of detecting a mycobacterial infection is based on detection of antibodies against LAM in a blood or serum sample from a patient (Theuer CP, Chaisson RE, Elias D (1989), Am. Rev. Respir. Dis. 139 (4, Part 2) : A 395).

20 However, when antibodies against a bacterial antigen are used for diagnosing it is possible that a past infection and/or vaccination rather than an on-going infection or disease is detected.

The ability to diagnose an on-going mycobacterial infection or disease at an
25 early stage could be life-saving for especially immunosupprimized patients, e.g. HIV infected patients. Mycobacterial diseases can be therapeutically treated by administration of certain antibiotics, such as Rifampicin, Etambutol, and Isoniazid.

30 It is also important to be able to monitor the effects of therapeutic treatment of a mycobacterial disease in a patient, especially since it is known that there are several mycobacterial strains which are resistant to antibiotics.

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An immunoassay kit for diagnosing a mycobacterial disease in a patient will be a useful tool in this context

5 **Description of the invention**

It has now surprisingly been found that lipoarabinomannans (LAM), and arabinomannans (AM), derived from *Mycobacterium tuberculosis* (TBC) and *Mycobacterium avium* complex (MAC), can be detected in samples of feces, sputum or urine from infected patients. It will now be possible to directly detect components of such bacteria and to determine the relative amounts of these mycobacterial antigens in samples of body fluids excreted by a patient, thus reflecting an on-going infection or disease in said patient.

15 Since LAM and AM are stabile at normal temperatures for sterilization (e.g. 60-100°C) the samples of feces, sputum or urine can be heat sterilized as a pretreatment, thus avoiding undeliberate contact with e.g. HIV infected body fluid, which is a great advantage for laboratory personnel.

20 Thus, the present invention is directed to a method of diagnosing a mycobacterial disease in a patient typically caused by *Mycobacterium tuberculosis* (TBC) or *Mycobacterium avium* complex (MAC), wherein, in a sample of feces, sputum or urine from said patient, the presence of a mycobacterial antigen selected from the group consisting of lipoarabinomannans (LAM), arabinomannans (AM), and fragments of LAM and AM, is determined.

25 Said determination is preferably performed by the use of polyclonal or monoclonal antibodies directed against said mycobacterial antigen, and of an assay detecting antigen/antibody complexes formed.

30

Examples of suitable assays which can be used are enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), and immunoblotting.

In an advantageous embodiment of the invention the sample of feces, sputum or urine is pretreated by heat sterilization.

5 The invention is further directed to an immunoassay kit, which comprises optionally labeled polyclonal or monoclonal antibodies directed against a mycobacterial antigen selected from the group consisting of lipoarabinomannans (LAM), arabinomannans (AM), and fragments of LAM and AM.

10 The labels of the antibodies are selected in agreement with the diagnostic method to be used, e.g. an enzyme label when ELISA is to be used etc.

In a preferred embodiment of the invention said antibodies directed against said mycobacterial antigen has been coupled to a carrier. The carrier may be a solid
15 support such as a plastic or glass surface, a membrane of e.g. derivatized carboxy cellulose, filter of e.g. Nylon or the like.

The immunoassay kit may additionally comprise
an optionally labeled second monoclonal or polyclonal antibody,
20 a positive control,
a negative control, and
optionally buffer solution(s) and/or washing solution(s).

Instructions for use will be provided with a kit according to the invention.

25 The present invention is further directed to a method of monitoring the effects of therapeutic treatment of a mycobacterial disease in a patient typically caused by *Mycobacterium tuberculosis* (TBC) or *Mycobacterium avium* complex (MAC), wherein, in a sample of feces, sputum or urine from said patient, the presence
30 and amount of a mycobacterial antigen selected from the group consisting of lipoarabinomannans (LAM), arabinomannans (AM), and fragments of LAM and AM, is determined at appropriate stages of said therapeutic treatment.

The monitoring will be performed by using the method of diagnosing a mycobacterial disease in a patient according to the invention at several, appropriate stages of therapeutic treatment of a patient, such as prior to
5 administration of e.g. an antibiotic, and then 3, 5, 10 and 20 days after the first administration etc. It will then be possible to evaluate the effects of the therapeutic treatment by comparing the amounts of detected mycobacterial antigen at the different stages of treatment.

10 The invention will now be further illustrated by the following specific, but not limiting, experiments.

Sandwich enzyme-linked immunosorbent assay (sandwich-ELISA)

15 A sandwich ELISA is used for the detection of mycobacterial antigens in a sample of urine from a tuberculosis patient.

The wells of a microtiter plate were coated with 100 µl of (mono or polyclonal) antibodies against lipoarabinomannan (LAM) per well. After overnight incubation
20 at room temperature, the unbound antibody was removed and the remaining free binding sites of the wells were blocked by 200 µl 0.5% casein for 1 h at 37°C. Excess casein was removed, the plate was washed 3 times with washing buffer (0.05% Tween 20 in PBS) and 100 µl of urine samples were added. After incubation for 1 h at 37°C, the plate was then washed and incubated with 100
25 µl of corresponding Biotin-labeled antibody (Method of biotinylation of IgG -see J.Cell Biol. 73,783-788). The plate was then washed and incubated with 100 µl of Extravidin-alkaline phosphatase conjugate (1/5000 dilution) for 1 h at 37°C. Excess of conjugate was removed, the plate was washed and developed by adding 100 µl substrate solution, and the absorbance at 405 nm was recorded.

30

D tection of LAM in urine using Immunodyne ABC (dip-stick t st)

1- Spot load 1 μ l of capture antibody (anti LAM IgG) in PBS onto the membrane and air dry for 10 min.

5

2- Block the membrane with e.g. 0.5% casein in PBS for 30 min at room temperature.

10

3- Incubate the membrane with the urine sample for 30 min at room temperature.

4- Wash the membrane in 2x changes of wash solution (0.1% Triton X-100 in PBS)

5 min per wash. Rinse the membrane with PBS.

15

5- Add biotinylated anti-LAM IgG (5 μ g/ml in PBS), incubate the membrane for 30 min at room temperature.

20

6- Repeat wash procedure as in step 4.

7- Add Extravidin-alkaline phosphatase (1/10,000 dilution), incubate for 30 min at room temperature.

25

8- Rewash the membrane as in step 4.

9- Develop the membrane by the addition of substrate solution.

30

To test the hypothesis that LAM is excreted with urine a group of mice was injected with a cell wall homogenate (1 mg/mouse) extracted from *Mycobacterium tuberculosis*.

Control mice were given phosphate buffered saline (PBS). Urin samples were collected from the mice on the next day and analyzed by both catch-up ELISA

(sandwich ELISA) and dip-stick test. Only animals injected with the cell wall homogenate became positive in the test, and none of the controls. Both polyclonal and monoclonal antibodies against LAM have been used in this assay.

5

Urine samples from 20 patients with active tuberculosis and from 3 patients with both HIV and Mycobacterium avium complex (MAC) were analyzed; all became positive in the test. 18 healthy control patients were also analyzed and the results were negative. Matched patients with other diseases (30 patients) were also included in these studies, and interestingly some of these control patients (5 patients, positive in the assay of the invention) who initially were clinically asserted to be none TB showed upon follow-up to either have TB or a more or less recent history of TB.

10

15

Purification of lipoarabinomannan (LAM) from Mycobacterium tuberculosis

20

Purified LAM may be used as a positive control in the immunoassay of the invention, and as starting material for the preparation of monospecific polyclonal antibodies against LAM (which will be exemplified below).

25

Dry cell wall (5 g) from Mycobacterium tuberculosis is sonicated in Na-acetate buffer, pH 4.7, 5X3 min, followed by extraction with 80% phenol for 1 h at 70°C. After centrifugation for 30 min at 3500 rpm the phenolic phase is reextracted with water, the phenolic phase is discarded, and the aqueous phase is pooled with the aqueous phase from the centrifugation. The pooled aqueous phase is dialyzed against 3 x 5 liters of water overnight. Chromatography on octyl-Sepharose® (Pharmacia , Sweden) yields 5 mg (0.1%) of LAM.

30

Preparation and purification of antibodies against LAM (anti-LAM IgG)

Both monoclonal and polyclonal antibodies may be use in the immunoassay of the invention. The preparation of polyclonal antibodies starting with the bacterial

cell wall, and monospecific polyclonal antibodies starting with LAM are exemplified.

Polyclonal antibodies

- 5 Bacterial cell wall (0.5 g) from *Mycobacterium tuberculosis* is sonicated to homogeneity in PBS. A rabbit is immunized with 50 µg homogenate in FCA (Freund's Complete Adjuvant) per lymph node at an interval of 2 weeks. The rabbit is then bled, and the antiserum is collected. Purification of anti-cell wall IgG is achieved with Protein-A Sepharose® (Pharmacia, Sweden). Purification of
- 10 the anti-LAM IgG is achieved by affinity-LAM column chromatography yielding purified polyclonal anti-LAM IgG.

Monospecific polyclonal antibodies

- LAM (6.5 mg) is oxidized with 0.01 M NaIO₄ for 7 min at 4°C in the dark.
- 15 Excessive NaIO₄ is eliminated by addition of ethylene glycol. Fragments of LAM are separated by gel chromatography. Coupling of the fragments to aminoethyl Bio-Gel P-2 (Bio-Rad, USA) through reductive amination at room temperature, pH 8, for 5 days results in a 70% yield. Blockage of excessive amino groups was performed by acetylation with Na-acetate using a water soluble
- 20 carbodiimide such as EDAC at room temperature, pH 4.5, for 17 h, followed by washing and subsequent equilibration of the column with PBS. Affinity-LAM column yields purified monospecific polyclonal anti-LAM IgG, which can be used instead of monoclonal antibodies in the immunoassay of the invention.

CLAIMS

5

1. A method of diagnosing a mycobacterial disease in a patient typically caused by *Mycobacterium tuberculosis* (TBC) or *Mycobacterium avium* complex (MAC), **c h a r a c t e r i z e d** in that in a sample of feces, sputum or urine from said patient, the presence of a mycobacterial antigen selected from
10 the group consisting of lipoarabinomannans (LAM), arabinomannans (AM), and fragments of LAM and AM, is determined.
2. A method according to claim 1, wherein said determination is performed by the use of polyclonal or monoclonal antibodies directed against said
15 mycobacterial antigen, and of an assay detecting antigen/antibody complexes formed.
3. A method according to claim 2, wherein said assay is selected from the group consisting of enzyme-linked immunosorbent assay (ELISA), radioimmunoassay
20 (RIA), and immunoblotting.
4. A method according to any one of claims 1-3, wherein said sample of feces, sputum or urine is pretreated by heat sterilization.
- 25 5. An immunoassay kit for diagnosing a mycobacterial disease in a patient, which comprises optionally labeled polyclonal or monoclonal antibodies directed against a mycobacterial antigen selected from the group consisting of lipoarabinomannans (LAM), arabinomannans (AM), and fragments of LAM and AM.
30
6. An immunoassay kit according to claim 5, wherein said antibodies directed against said mycobacterial antigen has been coupled to a carrier.

7. An immunoassay kit according to claim 6 or 7, which additionally comprises
an optionally labeled second monoclonal or polyclonal antibody,
a positive control,
5 a negative control, and
optionally buffer solution(s) and/or washing solution(s).

8. A method of monitoring the effects of therapeutic treatment of a
mycobacterial disease in a patient typically caused by *Mycobacterium*
10 tuberculosis (TBC) or *Mycobacterium avium* complex (MAC),
c h a r a c t e r i z e d in that in a sample of feces, sputum or urine from said
patient, the presence and amount of a mycobacterial antigen selected from the
group consisting of lipoarabinomannans (LAM), arabinomannans (AM), and
fragments of LAM and AM, is determined at appropriate stages of said
15 therapeutic treatment.

INTERNATIONAL SEARCH REPORT

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IPC 6 G01N33/569 G01N33/58

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 92 14156 A (DYNAGEN INC) 20 August 1992 see page 6 - page 8 see abstract	1-8
X	WO 92 14155 A (DYNAGEN INC ; UNIV COLORADO RES (US)) 20 August 1992 see page 4 - page 5 see page 35 - page 37	1-8
X	WO 92 14154 A (DYNAGEN INC) 20 August 1992 see claims 1,3,4,7	1-8
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☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

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NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
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Authorized officer

Cartagena y Abella, P

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ABSTRACTS OF THE GENERAL MEETING OF THE AMERICAN SOCIETY FOR MICROBIOLOGY, vol. 94, no. 0, 23 - 27 May 1994, page 174 XP002035609 S. N. CHO ET AL.: "Detection of Mycobacterium tuberculosis antigens in sputum samples from tuberculosis patients." see the whole document ---	1-8
X	YONSEI MEDICAL JOURNAL, vol. 31, no. 4, 1990, pages 333-338, XP002035610 S.N. CHO ET AL.: "Production of monoclonal antibodies to lipoarabinomannan-B and use in the detection of mycobacterial antigens in sputum." see the whole document ---	1-8
A	JOURNAL OF CLINICAL MICROBIOLOGY, vol. 30, no. 9, September 1992, pages 2415-2418, XP002035611 E SADA ET AL.: "Detection of lipoarabinomannan as a diagnostic test for tuberculosis." ---	
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP 97/01037

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WO 9214156 A	20-08-92	AU 1468692 A	07-09-92
WO 9214155 A	20-08-92	AU 1423892 A	07-09-92
WO 9214154 A	20-08-92	AU 1564592 A	07-09-92
EP 0273333 A	06-07-88	DE 3700049 A	14-07-88
		JP 63180859 A	25-07-88

Form PCT/ISA/210 (patent family annex) (July 1992)